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An extraordinary colonial spider community in Sulfur Cave (Albania/Greece) sustained by chemoautotrophy*

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Abstract

We report the discovery and detailed analysis of an extraordinary colonial spider assemblage in Sulfur Cave, a chemoautotrophic sulfidic ecosystem located on the Albania-Greece border. The colony, comprising an estimated 69,000 individuals of *Tegenaria domestica* (Agelenidae) and more than 42,000 of

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Prinerigone vagans (Linyphiidae), spans a surface area of over 100 m^2—representing the first documented case of colonial web formation in these species. Stable isotope analyses ($\delta^{13}C$ and $\delta^{15}N$) revealed that the trophic web sustaining this assemblage is fueled by *in situ* primary production from sulfur-oxidizing microbial biofilms then transferred through chironomid larvae and adults to higher trophic levels. Morphological and molecular data confirmed the identity of the two spider species and revealed that their populations in Sulfur Cave are genetically distinct from other populations. Regarding *T. domestica*, we found a seasonal pattern in fecundity, with significantly larger egg clutches in early summer. Microbiome analysis of this species also revealed a lower Shannon diversity in the cave population compared with a surface individual captured nearby. Our findings unveil a unique case of facultative coloniality in this cosmopolitan spider, likely driven by resource abundance in a chemoautotrophic cave, and provide new insights into the adaptation and trophic integration of surface species in sulfidic subterranean habitats.

Keywords
food-web analysis, *Prinerigone vagans*, Sarandaporo Valley, stable isotope ratio analysis, *Tegenaria domestica*

Introduction

Sulfidic subterranean ecosystems are sustained by *in situ* primary production predominantly by chemoautotrophic sulfur-oxidizing microorganisms. These organisms utilize the hydrogen sulfide (H_2S) as electron donor, and atmospheric dioxygen (O_2) as terminal electron acceptor (Chen et al. 2009; Kumaresan et al. 2014). The resulting high amounts of autochthonous organic matter support abundant and diverse aquatic and terrestrial communities of invertebrates as well as a few vertebrate species (Engel 2007; Hutchins et al. 2016). Sulfidic cave ecosystems often harbor numerous endemic species (Brad et al. 2021; Sarbu et al. 2024), some of which exhibit physiological and biochemical adaptations allowing them to survive in highly selective environmental conditions such as H_2S toxicity, hypoxia, and very low pH values (Flot et al. 2014; Tobler et al. 2016; Borko et al. 2019). The first chemoautotrophy-based groundwater ecosystem was discovered in Movile Cave in 1986 (Sarbu 1990; Sarbu et al. 1996). Subsequent investigations have revealed similar ecosystems in various locations, including the Frasassi caves (Italy; Sarbu et al. 2000), Ayyalon Cave (Israel; Por et al. 2013), Melissotrypa Cave (Greece; Popa et al. 2020), Tashan-Chah Kabootari Cave (Zagros Mountains, Iran; Malek-Hosseini et al. 2023), the Sharo-Argun cave system (Chechen Republic; Chervyatsova et al. 2020; Antić and Turbanov 2022), and more recently, several sulfidic caves in Albania (Benassi 2024; Sarbu et al. 2024; T. Delić, personal communication) within the Sarandaporo Valley, situated on the border between Greece and Albania (Audy et al. 2022; Kovařík et al. 2023; Sarbu et al. 2024).

Sulfur Cave is located at the border of two countries: its entrance is in Greece, while its deep sections are in Albania (Fig. 1). Along with Atmos Cave and Turtle Cave, it forms a hypogenic subterranean network of large rooms and passages lodged in a narrow limestone outcrop that was cut by the Sarandaporo River to form the Vromoner Canyon (*Vromoner* means "Smelly water" in Greek). Springs located in the deep recesses of the cave feed a sulfidic stream which flows through the entire length of the main

cave passage and reaches the Sarandaporo River at the cave entrance. Its water displays a constant year-round temperature of 26 °C and a H_2S concentration as high as 65 mg l^{-1} (Audy et al. 2022), and no seasonal or diurnal variations in flow rate were recorded for the cave stream. Occasional flooding events in the Sarandaporo River are only felt in the cave entrance area and appear to have limited effects on the cave ecosystem. The atmosphere in the cave can reach concentrations of up to 14 ppm of H_2S (Audy et al. 2022). The sediments in the stream are covered by abundant white biofilms consisting of filamentous sulfur-oxidizing bacteria (e.g., *Beggiatoa* spp., *Thiothrix* spp.). The aquatic cave fauna in this stream is very abundant and includes oligochaetes, gastropods, chironomid larvae, coleoptera larvae and adults, and occasionally some fish. The terrestrial fauna is represented by numerous centipedes, terrestrial isopods, pseudoscorpions, mites, scorpions, spiders, springtails, chironomid adults, and beetles (Sarbu et al. 2024).

In the stream passage located close to the cave entrance, a dense swarm of adult chironomid flies fills the air in the immediate vicinity of the sulfidic stream, and a large portion of the cave wall is covered by a massive colonial spider web (Figs 1, 2).

The European subterranean spider fauna includes 512 species belonging to 20 families, with at least 192 species considered to be obligate troglobionts (Mammola et al. 2022). In Sulfur Cave, a large colonial spider web covering a significant portion of one of the cave walls was first reported by a team of speleologists from the Czech Speleological Society who explored the sulfidic caves of the Vromoner Canyon and collected samples of fauna (Audy et al. 2022). Taxonomic analysis by V. Ružička reported the presence of the following spider taxa in Sulfur Cave: *Tegenaria domestica* (Fourcroy, 1785) (Agelenidae), which was identified based solely on the examination of a few female specimens, *Metellina merianae* (Scopoli, 1763) (Tetragnathidae), *Kryptonesticus eremita* (Simon, 1880) (Nesticidae), and another unidentified nesticid species. Sarbu et al. (2024) subsequently identified the dominant spider species in the colony as *Tegenaria domestica* (Clerck, 1757) (Agelenidae) (Fig. 3), commonly known as the "Domestic House Spider" or "Barn Funnel Weaver". A dense population of *Prinerigone vagans* (Andouin, 1826) (Linyphiidae) (Fig. 4) was also found co-inhabiting the colonial web.

Parasocial or colonial behavior (i.e., cooperation in web building by sharing the same spatial framework) has often been reported in subtropical and tropical arachnid communities. However, to date the only documented case of sub-social behavior in a cave-dwelling spider is that of *Goeldia* sp., observed by Almeida-Silva et al. (2009) in a Brazilian cave. This involved limited cooperative behavior and does not meet the criteria for true coloniality. Therefore, no confirmed cases of coloniality—defined by stable, large-scale cooperative web structures—have been reported among cave spiders (Mammola and Isaia 2017). Spider colonies, sometimes exceeding 100,000 individuals, are often correlated with locally high abundance of food resources (Rypstra 1979; Uetz 1983; Uetz and Hodge 1990; Uetz and Hieber 1997). Within Central Europe, the araneid spider *Larinioides sclopetarius* (Clerck 1757) is one of the few species known to form colonies with aggregations of 60 to 200 individuals across multiple generations building interconnected orb-webs (Schmitt 2004). Colonial behavior has never been observed in any agelenid or linyphiid spiders.

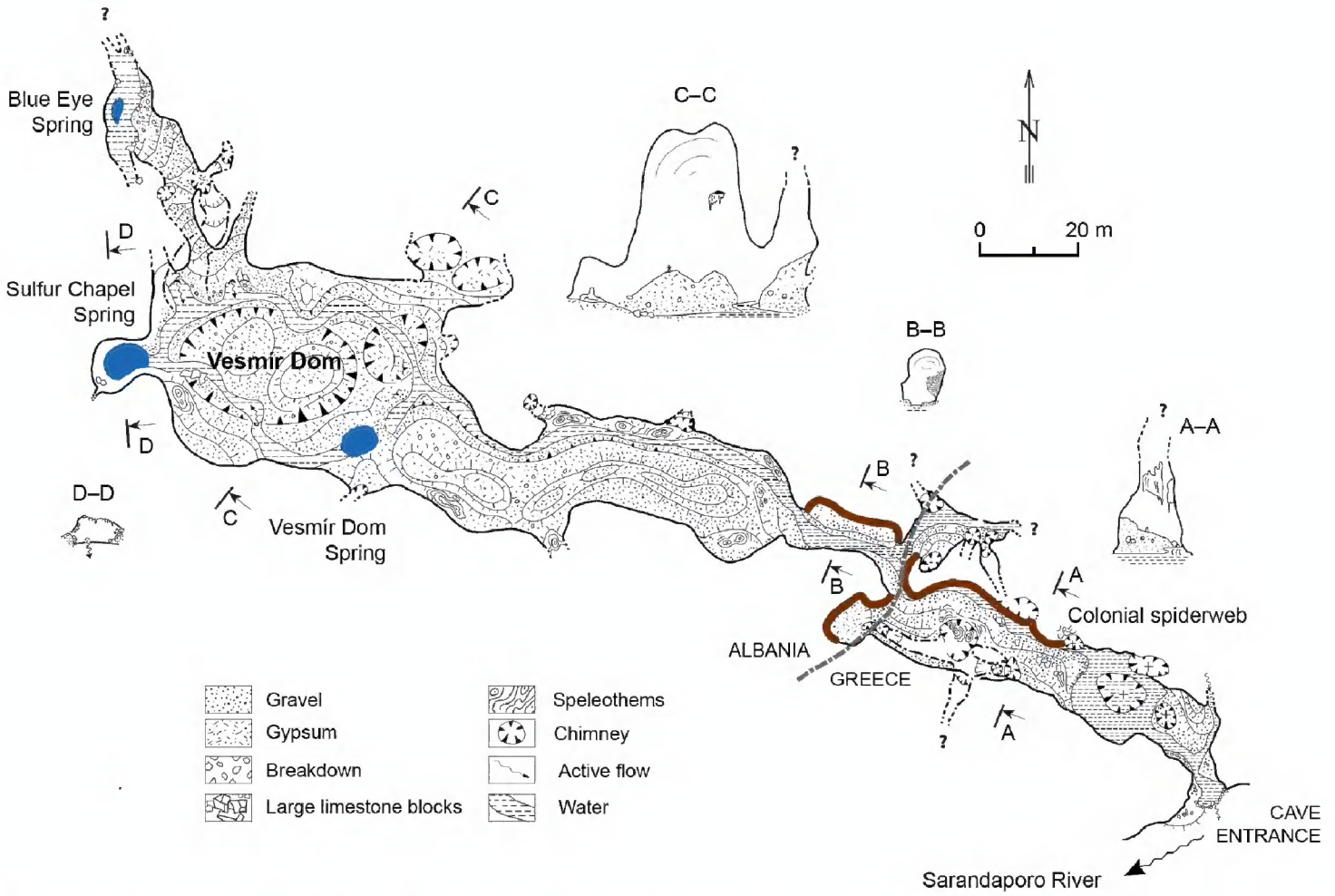

Figure 1. Plan of Sulfur Cave, with depiction of the main sulfidic springs (blue areas), and the large spider web in the vicinity of the cave entrance (brown areas) (modified from Audy et al. 2022 and Sarbu et al. 2024).

Food webs in caves are generally little studied since the trophic relationships between invertebrates and also between invertebrates and microorganisms are difficult to observe. One method to overcome those problems is the use of stable isotopes for understanding trophic levels in cave ecosystems. To analyze the trophic structure of cave animals and their food resources in Sulfur Cave, we therefore used stable isotope ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$). These ratios are often used to analyze trophic levels in invertebrate communities (Maraun et al. 2023) as they integrate animal dietary information over longer periods of time, which allows to reveal general characteristics of the trophic structure of food webs (Tiunov 2007; Nielsen et al. 2018). As ^{13}C is little enriched across trophic levels, it serves as an indicator for the base of the food web (Peterson and Fry 1987; Potapov et al. 2019a). In contrast, ^{15}N is enriched by about three delta units per trophic level thereby reflecting the trophic position of consumers (Post 2002; Pollierer et al. 2009; Potapov et al. 2019b).

This study presents a detailed characterization of the remarkable spider colony discovered within Sulfur Cave, focusing on its spatial distribution and dimensions, species composition and population density, and the trophic resources sustaining this unique assemblage. We also documented aspects of reproductive biology (egg number and size) of the dominant species within the colonial web, and examined the presence and distribution of other spider species within Sulfur Cave to understand habitat partitioning and potentially distinct food web dynamics.

Figure 2. The colonial spider web in Sulfur Cave, is home to a mixed colony of *Tegenaria domestica* and *Prinerigone vagans*. A. Side view; B. frontal view. The swarm of adult chironomids is visible near the cave stream. Photo A: Marek Audy.

Materials and methods

Spider specimens were manually collected from both large and small webs throughout Sulfur Cave. Voucher specimens were preserved in 70% ethanol and deposited in the collection of the Museum of Natural Sciences, University of Tirana, Albania, and the zoological collection of the Department of Life Sciences, Sapientia Hungarian University of Transylvania, Romania. For stable isotope analysis samples of invertebrates and of microbial biofilms covering the aquatic sediments in the cave stream were collected manually and stored at –80 °C prior to analysis.

Morphological identification of spiders from Sulfur Cave

Spider external morphology, with a specific focus on male and female genitalia was examined using a ZEISS Stemi 2000-C and a Nikon SMZ1270 stereomicroscope. The taxonomic identification was based on the resources available at Spiders of Europe (Nentwig et al. 2025).

Images of the female epigyne and male pedipalp of *T. domestica* were taken using a Nikon D5600 DSLR camera mounted on a Nikon SMZ1270 stereomicroscope (Fig. 3B, C). The specimens of *P. vagans* were photographed using a Kern ODC 825 microscope camera attached to a Kern OZL 464 trinocular stereomicroscope. Images of the female epigyne and male pedipalp were taken using a Kern ODC 825 microscope camera mounted on a Kern OBN 132 trinocular optical microscope (Fig. 3). Multifocal images were compiled using HeliconFocus software, digital images were processed using Photoshop software.

Molecular identification of spiders from Sulfur Cave and haplotype analysis

The morphological characterization of the spiders was complemented by DNA barcoding. DNA was isolated from the prosoma or legs of specimens collected in absolute ethanol and kept at 4 °C using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For *Tegenaria* spiders, we amplified and sequenced Folmer's fragment (COI) of the cytochrome *c* oxidase mitochondrial gene of 26 cave-collected individuals and 1 surface-collected individual (caught near the entrance of nearby Pixaria Cave) using the protocol described in Collard et al. (2025); Nanopore reads were subsequently assembled using amplicon_sorter (Vierstraete and Braeckman 2022). For the other spiders (namely, two specimens of *Kryptonesticus*, three specimens of *Prinerigone*, and two specimens of *Metellina*), a slightly shorter fragment of COI mitochondrial gene was amplified using the LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'; Folmer 1994) and chelicerate_reverse_2 (5'-GATGGCCAAAAAATCAAAATAAATG-3'; Barrett and Hebert 2005) primer pair. Those PCRs were performed in a 40 µL volume containing 1X Green GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 2.5 mM $MgCl_2$, 1X BSA, 0.1 mM dNTP, 1 U/µL GoTaq® Flexi DNA polymerase, 0.1 µM each primer and up to 10 ng/µL DNA. The cycling conditions consisted of an initial denaturation at 95 °C for 2 minutes followed by 5 cycles of denaturation at 94 °C for 40 seconds, annealing at 45 °C for 40 seconds, extension at 72 °C for 1 minute, 35 cycles of denaturation at 94 °C for 40 seconds, annealing at 51 °C for 40 seconds, extension at 72 °C for 1 minute and a final extension step at 72 °C for 5 minutes. Sanger sequencing was performed by Macrogen (Amsterdam, The Netherlands) and the resulting trace files were visually inspected and curated in Chromas v.2.6.6 (Technelysium Ltd., South Brisbane, Australia) and CodonCode Aligner v.3.7.1 (CodonCode Corporation, MA, USA). The resulting sequences were compared to the publicly available databases GenBank (Sayers et al. 2022) and Barcode of Life Data System (Ratnasingham and Hebert 2024).

Figure 3. *Tegenaria domestica* in Sulfur Cave. A Female next to a funnel shaped hole in the colonial spider web. B Female epigyne. C Male pedipalp with retrolateral view.

Sequences belonging to the same taxon or related taxa were downloaded and aligned on the MAFFT web server (Katoh et al. 2019; https://mafft.cbrc.jp/align-ment/server/index.html, accessed on the 29th of March 2025), sequence statistics and haplotype diversity (Hd) were estimated in DnaSP v.6 (Rozas et al. 2017) and haplotype networks were drawn in HaplowebMaker (Spöri and Flot 2020) using the median-joining algorithm (Bandelt et al. 1999).

Figure 4. *Prinerigone vagans* in Sulfur Cave. Male and male pedipalp (right). Female and female epigyne (left).

16S metabarcoding analyses

As *T. domestica* is the dominant species in the communal web, our preliminary microbiome analysis has focused on this species. It included six individuals caught at the cave entrance, 13 individuals caught in the first section of the spider wall encountered when walking from the entrance into the cave, and 13 individuals caught in the second section of the spider wall (both on the left bank of the stream); as well as one surface individual caught near the entrance of Pixaria Cave, a few kilometers away, as a comparison. Whereas the first section of the Sulfur Cave spider wall is characterized by numerous egg sacs visible on the web, the second section is almost devoid of egg sacs, suggesting that reproduction chiefly occurs on the first section. Whole opisthosomas

were used for DNA extraction, because the gut of spiders is not easy to separate from surrounding tissues. Although there are several organs located in the abdomen that contain microbial communities, the midgut is the largest one and it has been suggested in the literature that microbes associated with other organs do not influence the results significantly (see Kennedy et al. 2020 and references therein). DNA was extracted using the DNeasy PowerSoil Pro kit (Qiagen, UK), then 16S amplification and Nanopore sequencing was performed using the protocol described in Collard et al. (2025). The obtained reads were then analyzed in Emu (Curry et al. 2022) using its default database (Stoddard et al. 2015; O'Leary et al. 2016; Schoch et al. 2020).

Population density estimation of colony forming spiders and associated species

The density of the *T. domestica* population was estimated by counting individual funnel-shaped webs and then extrapolating these counts to the surface area occupied by the colony. A 15 × 15 cm quadrant was placed near the cave wall where the colony was present, at 30 randomly selected locations. At each location two high-resolution pictures were taken of each quadrat. The number of individual webs per quadrat, identified by the central funnel structure, including those overlapping the center of the web, was counted from the pictures. Note that this methodology is likely to slightly overestimate the total individual count in the colony, as some funnel webs may be abandoned/unoccupied. This survey was carried out in October 2023, April 2024 and March 2025. To calculate the surface area occupied by the colony, the length (L) and width (W) of the web-covered section of the wall were measured, and the area was computed using the formula: Area = L × W, assuming the colony forms an approximately rectangular patch. The *P. vagans* population density was estimated in March 2025 by counting individual spiders visible within 15 × 10 cm quadrants photographed at 30 random locations. The total estimated population size was extrapolated to half of the surface area determined for *T. domestica*, reflecting the observed spatial distribution pattern of *P. vagans*. The density of flies associated with the colony was estimated using a similar approach: individuals were counted within quadrats based on photographs, and the mean density was extrapolated to the total area occupied by the colony.

Reproductive biology of *Tegenaria domestica* - egg number and size analysis

A total of 86 egg sacs of *T. domestica* were collected from the surface of the colonial web during three sampling periods: June 2024 (n = 15), October 2024 (n = 40), and March 2025 (n = 31). Each egg sac was carefully opened using fine-tipped tweezers, and the number of eggs per clutch was recorded. Photographs were taken for further documentation. To assess egg size, 20 egg clutches were randomly selected, and three eggs from each clutch were measured using a Stage Micrometer Microscope calibrated to a precision of 0.01 mm.

Stable isotope analyses

For each species of invertebrate, a single individual was transferred into a tin capsule. Larger species were ground in a ball mill (Retsch Mixer Mill MM200, Haan, Germany), and 1–2 mg were weighed into the tin capsules. All samples were dried at 60 °C for 24 hours. Animal and biofilm samples were analyzed using a coupled system of an elemental analyzer (NA 1500, Carlo Erba, Milan, Italy) and a mass spectrometer (MAT 251, Finnigan, Bremen, Germany) adapted for the analysis of small sample sizes (Langel and Dyckmans 2014). Ratios of the heavy isotope to the light isotope ($^{13}C/^{12}C$, $^{15}N/^{14}N$, denoted as R) were expressed in parts per thousand, relative to the standard using the delta notation with $\delta^{13}C$ or $\delta^{15}N = (R_{sample}/R_{standard} -1) \times 1000$ (‰); R_{sample} and $R_{standard}$ being the respective target isotope ratio ($^{15}N/^{14}N$ or $^{13}C/^{12}C$, respectively). Vienna PD Belemnite limestone (V-PDB) and atmospheric nitrogen were used as standard for ^{13}C and ^{15}N, respectively. Acetanilide (C_8H_9NO, Merck, Darmstadt) was used for internal calibration. Details on the analysis are given in Reineking et al. (1993).

Statistical analyses

All statistical analyses were performed in R v.4.4.2 (R Core Team, 2024). To compare the number of eggs among sampling months, we conducted a one-way analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) post hoc test for pairwise comparisons. Graphical representations of the data were generated using the ggplot2 package v.2.3.5.2 (Wickham 2016). Tabular data files containing the sequences and associated metadata downloaded from the BOLD database were transformed using the dplyr package v.1.1.4 (Wickham et al. 2023) and the sequences were exported to fasta files using the *dat2fasta* function of the phylotools package v.0.2.2 (Zhang 2024). Finally, we calculated Shannon's diversity index for the microbiome of cave- and surface-dwelling *Tegenaria domestica* individuals using a custom code.

Results

Using both morphological and molecular analyses, we identified five spider species in Sulfur Cave. These include one colony-forming species (*Tegenaria domestica*), one colony-associated species (*Prinerigone vagans*), and three additional non-colony-forming or non-colony-associated species: *Metellina merianae* (Scopoli, 1763) (Tetragnathidae) (Fig. 8), *Lepthyphantes magnesiae* Brignoli, 1979 (Linyphiidae), and *Kryptonesticus eremita* (Simon, 1880) (Nesticidae).

The molecular analysis showed that the sequence alignments for *T. domestica* (n = 72 sequences), *K. eremita* (n = 11 sequences), *M. merianae* (n = 47 sequences), *P. vagans* (n = 20 sequences) varied in length from 613 bp to 658 bp. *Lepthyphantes magnesiae* was excluded from the analyses due to the lack of publicly

available COI sequence data for comparison. All the BOLD sequences used and their relevant metadata are provided in Suppl. material 1. The haplotype sequences generated in this study are deposited in GenBank under the accession numbers PV793491–PV793494, PV793496 and PX363529.

The haplotype networks (Fig. 5) of *T. domestica* (Fig. 5A), *K. eremita* (Fig. 5B), and *P. vagans* (Fig. 5C) show distinct haplotypes for Sulfur Cave populations compared to sequences available online. *Tegenaria domestica*'s haplotype network (Hd = 0.321) is dominated by a central haplotype present in samples from North America, Europe, Turkey, Pakistan, and New Zealand. All 32 *T. domestica* samples from Sulfur Cave shared the same haplotype, one mutation away from this common haplotype that was also sampled a few kilometers away from Sulfur Cave (in the vicinity of Pixaria Cave).

The haplotype network of *K. eremita* (Hd = 0.818) shows that the two cave individuals share a haplotype separated by three mutations from haplotypes found in Albania and Croatia. A similar situation is observed for *P. vagans* (Hd = 0.352); the three cave individuals sequenced shared the same haplotype, which is three mutations away from the common haplotype present in samples from France, Germany, Bulgaria, Egypt, Lebanon, and Pakistan. By contrast, *M. merianae* (Fig. 9D) presents a more complex, highly-reticulated haplotype network (Hd = 0.912) in which the two samples from Sulfur Cave belong to two distinct haplotypes, one of them shared with samples from Germany.

The spatial distribution of *T. domestica* in Sulfur Cave

The large spider colony in Sulfur Cave was mainly observed on the left bank of the sulfidic stream of Sulfur Cave, in a permanently dark zone, starting at approximately 50 m from the cave entrance. In this section of the cave, the passage is relatively narrow, and the ceiling is mostly low (Fig. 5). The location of the colonial spiderweb coincides with an area where an unusually dense swarm of small chironomid flies identified as *Tanytarsus albisutus* Santos Abreu, 1918 (a member of the *triangularis* group), hovering above the sulfidic stream that flows along the left cave wall.

Population density estimation of *T. domestica* and *P. vagans*

The estimated total surface area occupied by the spider colony was ~ 106 m^2. Based on the extrapolated quadrat counts, the total population size of *T. domestica* was estimated at 69,113 spiders with the mean density of 652 ± 149 individuals per m^2 (range: 178-2756). For *P. vagans* the estimated total number of individuals was 42,400, with a mean density of 823 ± 556 per m^2 (range: 133-2200). The density of the chironomid flies resting on the cave wall was estimated at 45,500 individuals per m^2. Based on this density and the observed spatial distribution pattern, where flies cover approximately half of surface area occupied by the spider colony, the total number of flies was extrapolated to be 2,414,440 individuals.

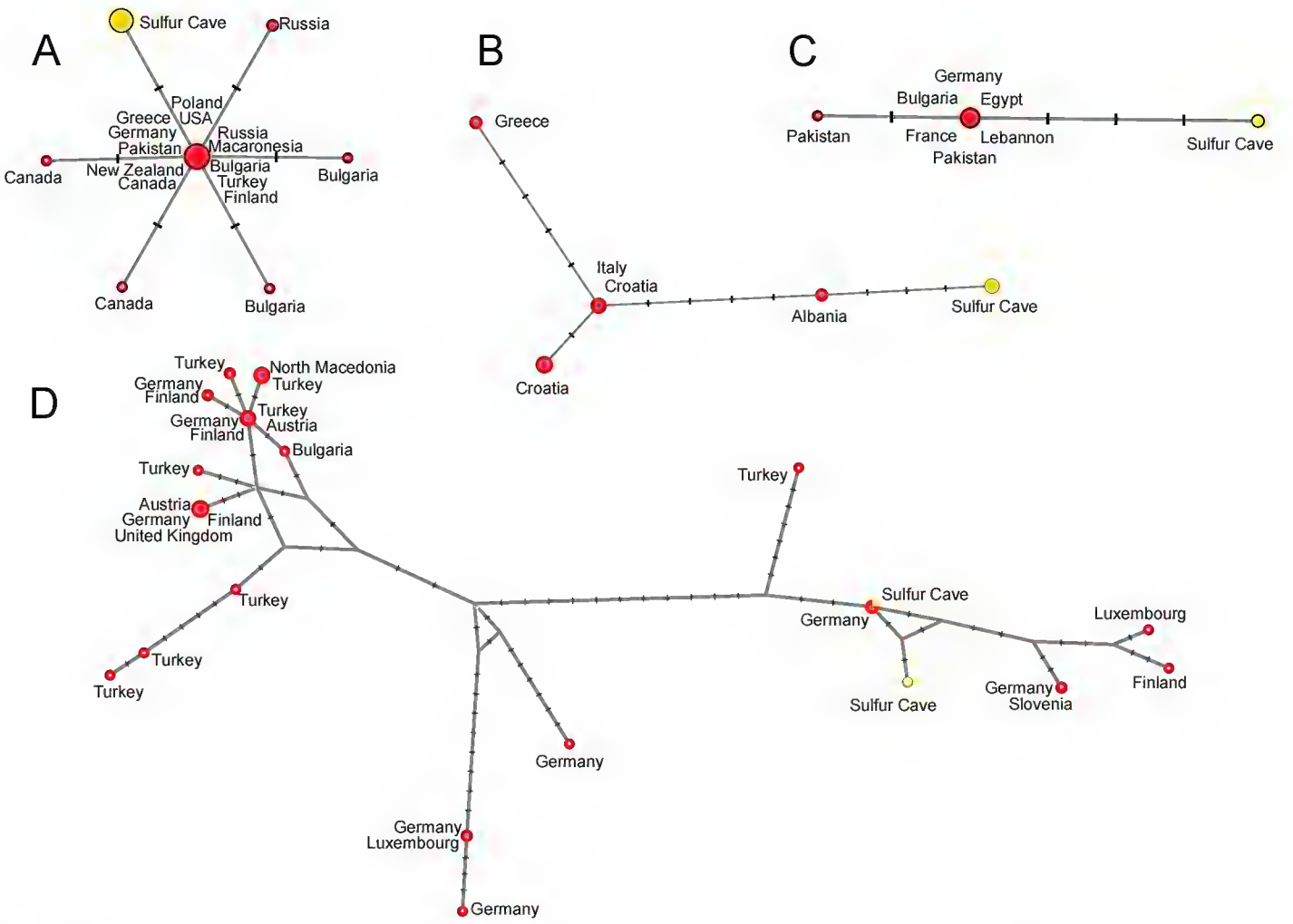

Figure 5. Haplotype networks of COI sequences for four spider species from Sulfur Cave separated into individual panels **A** *Tegenaria domestica* **B** *Kryptonesticus eremita* **C** *Prinerigone vagans* **D** *Metellina meriana*. The haplotypes from Sulfur Cave are shown in yellow. The circle size is proportional to the number of sequences in each haplotype and the number of mutations between haplotypes are denoted by hash marks.

Reproductive biology (egg number and size) of *T. domestica*

The mean number of eggs per *T. domestica* clutch was 16.15 ± 0.62 SD (range: 6–33 eggs) with significant seasonal variations (*ANOVA*: $F_{[2,83]} = 69.42$, $p < 0.001$). Tukey's post hoc test showed that clutches collected in June (mean ± SD: 26 ± 3.12) contained significantly more eggs compared to those collected in October (14.5 ± 3.6) (mean difference: 11.550, 95% Confidence Interval (CI) = [8.966, 14.133], $p < 0.001$) and March (13 ± 3.73) (mean difference: 12.419, 95% CI = [9.735, 15.103], $p < 0.001$), while no significant difference was found between October and March (mean difference: 0.869, 95% CI = [-1.172–2.911], $p = 0.569$) (Fig. 6). The mean egg size was 0.53 ± 0.05 mm, with values ranging from 0.4 to 0.6 mm.

Food-web analysis

The $\delta^{15}N$ and $\delta^{13}C$ values in adult *Tanytarsus albisutus* (Diptera, Chironomidae), as well as in both agelenid and linyphiid spiders inhabiting the colony, ranged from –2‰ to –10‰, in contrast to conspecifics or ecologically similar surface-dwelling taxa, which showed values between 1‰ and 4‰ (Fig. 7).

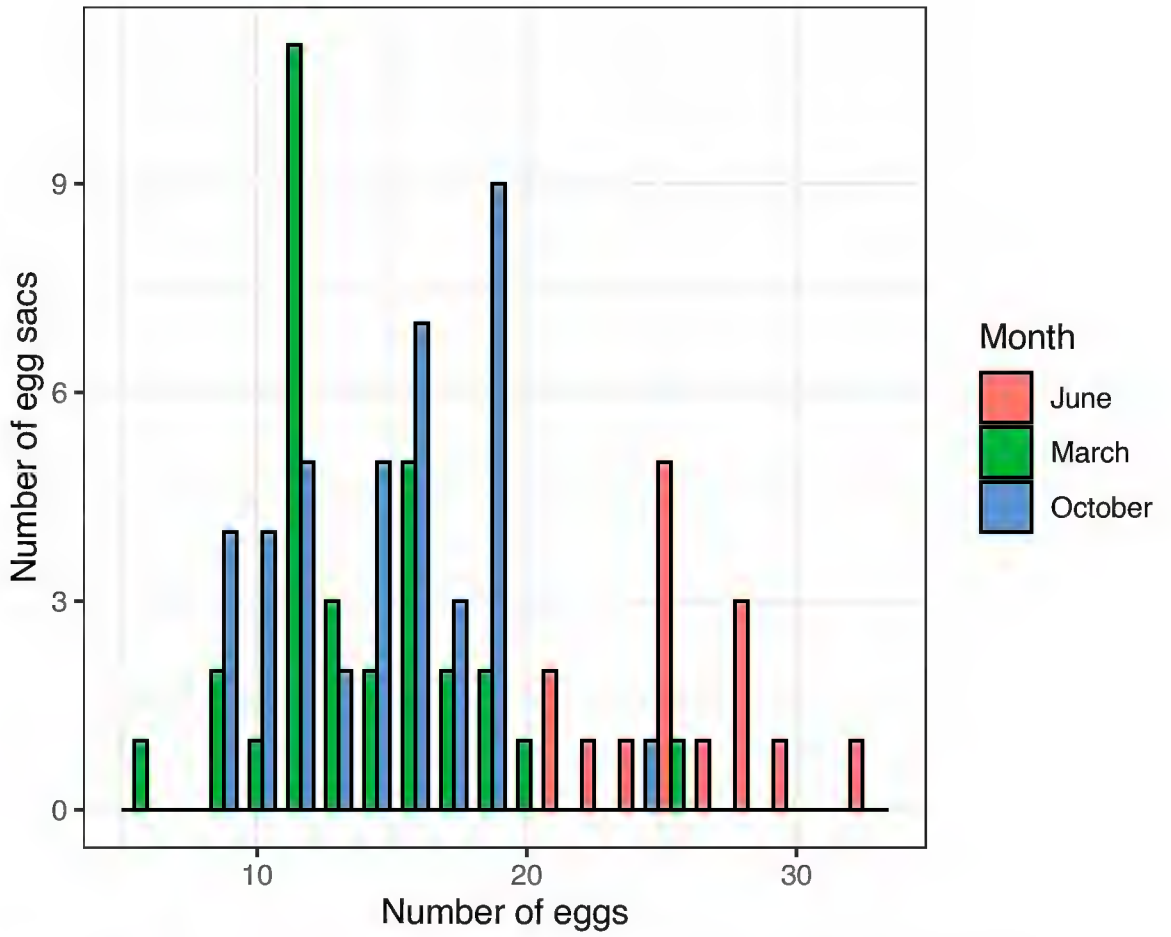

Figure 6. Monthly variation in the number of eggs per egg sac in *Tegenaria domestica*.

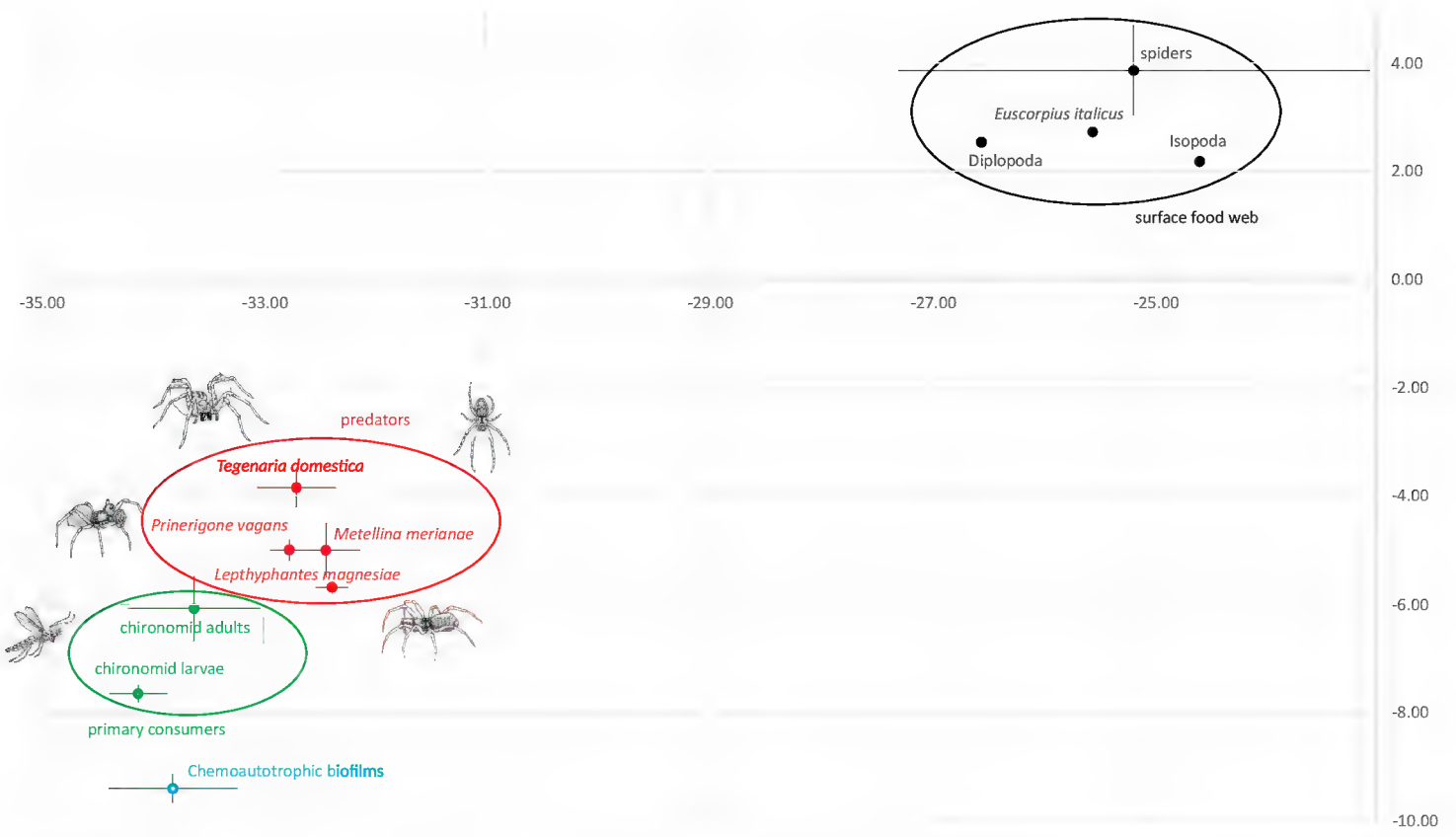

Figure 7. Mean (+ standard deviation) of $\delta^{13}C$ and $\delta^{15}N$ values of four spider species (*Tegenaria domestica*, *Metellina merianae*, *Prinerigone vagans*, *Lepthyphantes magnesiae*, Araneae), their potential food resource (the non-biting midge *Tanytarsus albisutus*) and the basal food resource (biofilm) of the cave food web in Sulfur Cave located at the border between Albania and Greece (animal drawings by Svenja Meyer). 'Surface spiders' include representatives of the families Lycosidae, Salticidae, and Pholcidae.

Microbiomes of cave- and surface-dwelling *T. domestica*

In the one surface individual of *T. domestica* analyzed (collected near Pixaria Cave), we detected 30 different bacterial genera. By contrast, the microbiome of *T. domestica* from Sulfur Cave showed a much lower bacterial diversity (Fig. 8). The mean Shannon diversity index was 0.82 for Sulfur Cave individuals (standard deviation: 0.25), in contrast to 2.99 for the surface-dwelling individual. A large proportion of the bacterial reads in Sulfur Cave individuals originated from intracellular symbionts, primarily *Mycoplasmopsis*, *Mycoplasma*, and *Wolbachia*.

Discussion

The spider colony

We report the discovery and detailed analysis of an extraordinary colonial spider assemblage in Sulfur Cave. Morphological examinations and molecular analyses identified *Tegenaria domestica* as being responsible for weaving the enormous colonial web. *Prinerigone vagans* (Andouin, 1826), a small linyphiid spider, was also observed co-habiting the web of the much larger agelenids. The colony covers a surface area of over 100 m^2 and represents the first documented case of colonial web formation in these species, comprising an estimated 69,000 individuals of *T. domestica* and more than 42,000 of *P. vagans*. The methodology used to estimate spider density may lead to an overestimation due to the presence of abandoned funnel webs that are difficult to distinguish from those in use.

Tegenaria domestica is a cosmopolitan species with a global distribution (Nentwig et al. 2025; World Spider Catalog 2025). It is a nocturnal and synanthropic spider usually found close to human habitations, such as in buildings, under stones, in dark recesses, i.e. closets and basements, with adults present year-around (Nentwig et al. 2025). While frequently found in caves across Europe (Mammola et al. 2018, 2022) and

Figure 8. Comparison of the Shannon diversity index of the microbiome of the opisthosoma of one surface individual of *Tegenaria domestica* caught near Pixaria Cave vs. 29 individuals of the same species from Sulfur Cave (three Sulfur Cave individuals with insufficient number of reads were excluded from the analysis). S0 individuals were caught at the entrance of Sulfur Cave, S1 on the first section of the spider wall and S2 and the second section.

considered a characteristic species for Balkan caves (Bristowe 1958; Deltshev et al. 2011), prior literature did not describe *T. domestica* exhibiting gregarious behavior and forming large colonial webs. The species typically builds a web resembling an open-cut funnel, with a tubular retreat open on both ends and its edge widening on one side to form a sheet-like capture surface (Nentwig et al. 2024). On a close examination, the large colonial web appears to be the result of joining numerous individual funnel-shaped webs, each strategically positioned in a spot of abundant trophic resource availability. Sections of the web may detach from the wall under their own weight. The colonization of Sulfur Cave by *T. domestica* was most likely driven by the abundant food resources represented by the dense swarm of chironomids thriving in the cave.

Prinerigone vagans, the co-habiting spider species within the colony, apparently did not elicit a predatory response from *T. domestica*. We hypothesize that the absence of light impairs the visual detection capabilities of *T. domestica*, rendering the small *P. vagans* inconspicuous, particularly given *P. vagans* stationary ambush predatory strategy with brief movements when prey items are in close proximity within the web. Although we did not conduct a specific study to quantify seasonal variations, our observations indicate no discernible changes in the species composition and abundance of the two spider species that co-habit the large colony in Sulfur Cave. Comparable densities of these spiders were observed throughout the year during our field trips in May, June, and November 2023, in April, July, and October 2024, and in March 2025. Sustaining a predator colony of this size inside a cave requires a substantial and consistent supply of food and this is unlikely to be met solely by allochthonous resources from the surface, where resources become rather scarce during the cold season.

Other spider species in Sulfur Cave

Metellina merianae (Scopoli, 1763) (Tetragnathidae) (Fig. 9) has been previously reported from Sulfur Cave (Audy et al. 2022). The juveniles prey upon collembola, while the adults feed on the two species of chironomids: the *Tanytarsus albisutus* and the larger *Chironomus riparius* (Meigen, 1804), mostly present in the deeper sections of the cave. *M. merianae* builds solitary orb webs and was not observed within the colonial web, yet it is quite abundant in close proximity to the colony where the dense chironomid swarm occurs. Specimens observed close to the colonial web appear to exhibit larger body size, presumably due to the readily available and abundant food resources. However, this species was observed throughout the cave, including the areas covered by gypsum crusts that appear rather dry. As a troglophile species (Mammola et al. 2018, 2022, Nentwig et al. 2025), *M. merianae* appears to be versatile and able to adapt to both wet and dry cave habitats.

Lepthyphantes magnesiae Brignoli, 1979 (Araneae: Linyphiidae) was observed on the cave wall opposite the colonial web. It builds individual webs and it shares the habitat with a pseudoscorpion species belonging to the genus *Neobisium*. Both these predator species have been noticed in the humid areas at the base of walls, where they appear to feed on collembola and chironomid flies. *Lepthyphantes magnesiae* is considered a rare species endemic to the Balkan Peninsula.

Figure 9. *Metellina merianae.* Female (left) and male (right) in individual webs on the cave wall.

In the deep recesses of the cave, far from the cave entrance and the colonial web, two additional web-building spider species were also observed: *Kryptonesticus eremita* (Simon, 1880) (Nesticidae), previously reported from Sulfur Cave by Audy et al. (2022), and a very small, blind, and depigmented species of *Cataleptoneta* (Leptonetidae). Both *K. eremita* and *Cataleptoneta* sp. were present in large numbers but were spatially limited to areas where the cave walls were moist and free of gypsum crusts.

Genetic and geographic patterns of spider species in Sulfur Cave

Haplotype networks revealed both similarities and contrasts among the spider species found in Sulfur Cave. For *T. domestica*, *K. eremita*, and *P. vagans*, the cave individuals shared distinct haplotypes not found in broader regional datasets, though all were closely related, i.e. within three mutations, to widespread haplotypes from Europe, the Middle East, or Asia. In contrast, *M. merianae* showed a more complex and reticulated haplotype network, with two individuals from Sulfur Cave belonging to different haplotypes, one shared with German samples. Despite the geographical proximity of some samples to Sulfur Cave (e.g. from Slovenia, Bulgaria, North Macedonia), they were quite distant in the network. This suggests that *M. merianae* is both more geographically mobile and more genetically diverse than the other spider species found in Sulfur Cave.

Reproductive biology of *T. domestica*

The reproductive effort, measured as the number of eggs per sac, differed from values reported in the literature for surface-dwelling conspecifics. Females of *T. domestica* are known to lay six to eight egg sacs at an interval of 20–25 days, with the first cocoon containing up to 100 eggs, followed by smaller successive clutches (Trabalon et al. 1992). Numerous egg-sacks were observed in the large spider web, but their number was not estimated as the multi-layered web made it impossible to count the egg clutches. The underlying factors contributing to a potential reduction in clutch size in the Sulfur Cave population of this species are yet to be determined, although environmental factors such as perpetual darkness and sulfidic conditions have been shown to be associated with reduced fecundity and increased offspring size (Riesch et al. 2010).

Microbiomes of cave- and surface-dwelling *T. domestica*

At the microbiome level, we found that, by contrast to one surface-dwelling individual of *T. domestica*, collected in the vicinity of the cave, in which approximately 30 different bacterial genera were detected, the cave-dwelling individuals investigated exhibited markedly lower bacterial diversity. In these individuals a large proportion of the reads originated from intracellular bacterial symbionts, such as *Mycoplasmopsis*, *Mycoplasma* and *Wolbachia*. Although the results are quite preliminary and do not allow to distinguish the relative contributions of the various parts of the opisthosoma to the measured diversity, it suggests that the population of *T. domestica* from Sulfur Cave presents a much-reduced microbial diversity than surface ones.

Alternate food web in Sulfur Cave

Preliminary observations indicate that in the deep cave sections, the food web is based on chemosynthetic carbon fixation in terrestrial microbial biofilms that cover the moist cave walls. These biofilms consist of sulfur-oxidizing microorganisms (unpublished). Numerous specimens of *Graeconiscus* sp. (Isopoda, Trichoniscidae) and dense populations of collembola appear to feed on this cave-wall microbiome, forming the base of a food web that supports two species of spiders and other small terrestrial predators such as centipedes, pseudoscorpions, mites, and beetles (Sarbu et al. 2024).

Conclusions

Similarly to other sulfidic subterranean ecosystems based on autochthonous food production by chemoautotrophic microorganisms, Sulfur Cave in the Vromoner Canyon located on the border between Greece and Albania contains exceptionally abundant and diverse invertebrate communities that thrive in total darkness. The most impressive occurrence in this cave is a large colonial spider web that covers an estimated

100 m^2 of cave wall and hosts ~69,000 specimens of *T. domestica* and ~42,000 specimens of *P. vagans*. These are both surface species that have never been reported to form colonies, and molecular evidence suggests that the Sulfur Cave population does not exchange individuals with the surface. Preliminary analyses of the microbiome of *T. domestica* also suggest that the cave population of this species is isolated from surface ones and presents a depauperate microbial diversity.

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Supplementary material 1

BOLD sequences used within this study

Authors: Andrei Ştefan

Data type: xlsx

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